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Dated: 11/28/05Signature: Kathryn Lugo
(Kathryn Lugo)Docket No.: AREX-P03-004
(PATENT)**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Patent Application of:
Madiyalakan *et al.*

Application No.: 09/376,604

Confirmation No.: 6693

Filed: August 18, 1999

Art Unit: 1642

For: THERAPEUTIC COMPOSITIONS THAT
ALTER THE IMMUNE RESPONSE

Examiner: G. B. Nickol

DECLARATION UNDER 37 C.F.R. § 1.132

MS AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Birgit C. Schultes, Ph.D., of 12 Monadnock Road, Arlington, MA, hereby declare and state as follows:

1. I am Senior Director of Research at Unither Pharmaceuticals, and an inventor on the present application. I have been conducting research in the field of tumor immunology for approximately 16 years. A copy of my *curriculum vitae* is enclosed with this Declaration.
2. I have read the Office Action issued by the U.S. Patent and Trademark Office on July 29, 2005 in the above-identified patent application, and U.S. Patent No. 5,532,159 naming Webb et al. as inventors ("*Webb*"). I have also reviewed the pending claims and the specification of the present application.

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3. I understand that the Examiner has taken the position that claims 113, 117-120, 123, 131-135, 137-139, 141-144, 170-174, 180-182, 185, 190, 193, 195, 197-204, 206-209, 235-239, 241-242, 251, 260-261, 264-265, 268-269, and 272-273 of the present application are anticipated by *Webb*. As will be discussed in further detail below, *Webb* discloses that the administration of an anti-OFP antibody to Sprague Dawley rats and NIH Swiss Nude mice bearing tumors resulted in a reduction in tumor size within one day of the administration of the antibody.

4. I understand that the pending claims require the use of an antibody (or antigen binding fragment thereof) which binds to a multi-epitopic antigen present in a host's serum, and forms an immune complex with the antigen, such that a host T cell or humoral immune response against the antigen in the immune complex is elicited. I understand that the Examiner has taken the position that the functional limitation of eliciting a host T cell or humoral immune response against the antigen in the immune complex is an inherent property of the antibody used in *Webb*. This is incorrect. Not all antibodies elicit an effective host T cell or humoral immune response against the antigen to which they bind. As discussed in detail below, there is no evidence that the anti-OFP antibodies administered by *Webb* elicited an effective host T cell or humoral immune response against the OFP antigen. Moreover, all of the evidence indicates that the administration of the anti-OFP antibody to animals by *Webb* did not elicit a host T cell or humoral immune response against the antigen.

5. OFP (oncofetal protein) is a polypeptide that is secreted from cancer cells. *Webb* states that "[t]umors treated with a single dose of the monoclonal antibodies against OFP are markedly reduced in size, and leukemic populations of cells treated with a single dose of monoclonal antibodies against OFP are significantly decreased in numbers" (col. 2, lines 49-53). OFP is immunosuppressive. *Webb* states that it is their belief that "by sequestering or removing OFP via the monoclonal antibody, the patient's immune defense against tumors is released from impairment allowing a more efficient and natural rejection of cancer" (col. 2, lines 56-60; *see also* col. 3, lines 61-65).

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6. *Webb* discloses the administration of a single 100 µg to 165 µg dose of anti-OFP antibodies to 50 day old female Sprague Dawley rats bearing DMBA-induced tumors (columns 11-12). As shown in Table 1, in essentially all cases, the tumor volume decreased within one day after the injection of the antibody. (See col. 11, lines 9-31 and Table 1.) The fact that in essentially all cases the tumor volume decreased within one day after the injection of the antibody indicates that the antibody either has a direct effect on the tumor or unleashes an already existing but suppressed immune response against the tumor. In contrast, for the reasons discuss below in ¶ 8, induction of an immune response to OFP using an anti-OFP antibody would require at least 5-7 days to elicit an initial T cell response and/or at least 5-20 days to elicit a humoral immune response. (Table 1 of *Webb* indicates that in approximately 3 out of 46 cases the administration of the anti-OFP antibody did not result in a decrease in tumor volume until day 6 after the administration of the antibody; however, these results are likely due to experimental variability and do not suggest that the antibody is eliciting an immune response against OFP. As discussed herein (¶¶ 8-1), the evidence as a whole indicates that these antibodies do not elicit an immune response against OFP.)

7. *Webb* also discloses the administration of a single 10 µg dose of an anti-OFP antibody to six (6) month old NIH Swiss Nude mice bearing tumors induced by the injection of human breast adenocarcinoma cells (col. 12, lines 58-65). Figure 1 shows that the anti-OFP antibody was administered on day 7, and that a reduction of tumor size was observed immediately (within days 7-10).

8. It is well known that it takes more than one day to elicit an effective T cell or humoral immune response against an antigen. (See, e.g., Peakman and Vergani, *Basic and Clinical Immunology*, Churchill Livingstone, 1997, pp. 41-42 (Exhibit A), stating that the primary "antibody response is detected 5-10 days after antigen injection, rises over the next 10-20 days, and then declines to a low level without ever completely disappearing." See also Thaler et al., *Medical Immunology*, Ed. J.B. Lippincott Company, 1977, pp. 23-25 and 28 (Exhibit B) teaching that injection of antigen leads to blast-cell activity in the T-cell paracortical areas by two days, and several days later, follicular activity is noted and germinal centers are formed (i.e., beginning stages

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of a host T cell response; see left column of page 23); and Durkin and Waksman, *J. Immunol.*, 1975; 115(1): 170-176 (Exhibit C) teaching that lymph node cells such as T lymphocytes in response to the OA antigen first appeared in culture at day 5 and peaked at day 9 to 12 days (see Figures 1 and 2 and description at right column of page 172). Accordingly, the fact that *Webb* observed an anti-tumor effect within one day of the administration of the anti-OFP antibodies indicates that the antibodies are not causing this effect by eliciting a host T cell or humoral immune response against the antigen.

9. Furthermore, six (6)-month old NIH Swiss Nude mice lack functional T cells and consequently have an impaired immune system, and would not have been able to raise an effective host T cell and an effective humoral immune response against an antigen. (Although nude mice could raise IgM antibodies, these antibodies cannot elicit an effective humoral immune response because nude mice lack helper T lymphocytes and lack a functional complement system.) Accordingly, the fact that *Webb* observed an anti-tumor effect in these mice is further evidence that the anti-OFP antibodies are not causing this effect by eliciting a host T cell or humoral immune response against the antigen.

10. *Webb* also discloses that the administration of a mixture of anti-OFP antibodies and excess OFP to rats bearing DMBA-induced tumors did not inhibit and, in fact, appeared to accelerate tumor growth (col. 11, lines 53-59). The pending claims recite the use of an antibody (or antigen binding fragment thereof) which forms an immune complex with a multi-epitopic antigen present in a host's serum and elicits a host T cell or humoral immune response against the antigen in the immune complex. If the anti-OFP antibody disclosed in *Webb* were exerting its anti-tumor effect by forming an immune complex with OFP and eliciting an immune response against the antigen in the immune complex as required by the claims of the present application, one would have expected that the *in vitro* generated immune complexes of anti-OFP antibodies and OFP antigen would have elicited an effective immune response in the animal resulting in superior inhibition of tumor growth as compared to the anti-OFP antibody alone. This did not occur.

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11. As discussed above (¶6), *Webb* discloses that the administration of anti-OFP antibodies to Sprague Dawley rats bearing DMBA-induced tumors decreased tumor volume within one day of antibody administration, and that tumor volume remained suppressed for 8-22 days (col. 11, lines 27-31). Further, *Webb* discloses the administration of anti-OFP antibodies to rats having transplantable rat hepatoma 7777 tumors also decreased tumor volume within one day of antibody administration (col. 12, lines 43-51). However, as shown in Table 1, col. 15-17 and recited in col. 12, lines 50-51, in both instances, some tumors appears to have grown (increased in tumor volume) after six days of antibody administration. If the anti-OFP antibody disclosed in *Webb* were exerting its anti-tumor effect by eliciting an immune response to OFP as required by the claims of the present application, one would have expected that tumor shrinkage would continue after 5-6 days of treatment and even accelerate at a time point the immune response is expected to peak. This observation suggests that the anti-OFP antibody administered by *Webb* did not mediate tumor regression via eliciting an immune response to OFP.

12. In summary, all of the evidence shown in *Webb* is consistent with *Webb*'s belief that the anti-OFP antibodies result in a reduction of tumor size by removing the immunosuppressive OFP antigen from the blood stream, and inconsistent with the Examiner's position that the anti-OFP antibodies inherently possess the functional property of eliciting a host T cell or humoral immune response against the OFP antigen.

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13. I state that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: Nov. 18, 2005Signed: 
Dr. Birgit C. Schultes

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EMPLOYMENT HISTORY

2004-present	Senior Director, Research Unither Pharmaceuticals, Inc., Wellesley, MA, USA
2002-2004	Vice President, Research AltaRex Corp., Waltham, MA, USA
2000-2002	Director, Research and Clinical Immunology AltaRex Corp., Waltham, MA, USA
1998-2000	Director, Preclinical Research AltaRex Corp., Edmonton, AB, Canada
1996-1998	Scientist and Senior Scientist, Research & Development AltaRex Corp., Edmonton, AB, Canada
1995-1996	Scientist, Research & Development Biomira Inc., Edmonton, AB, Canada
1994-1995	Postdoctoral Fellow, Research & Development Biomira Inc., Edmonton, AB, Canada
1990-1993	Supervisor, Clinical Chemistry, Tumor Marker Laboratory Clinic of Nuclear Medicine, University of Bonn, Germany

EDUCATION

1989-1993	Ph.D., summa cum laude, Cell Biology/Immunology University of Bonn, Germany
1982-1989	M.Sc., Biology (Genetics, Cell Biology, Biochemistry) University of Bonn, Germany and University of Cologne, Germany, Thesis on purification and characterization of an enzyme that regulates DNA metabolism.

MEMBERSHIP OF SCIENTIFIC SOCIETIES

American Association for Cancer Research
American Association of Immunologists
American Society of Photobiology
Society of Tumour Targeting

PROFESSIONAL EXPERIENCE***UNITHER PHARMACEUTICALS, WELLESLEY, MA (2004-PRESENT)*****Senior Director, Research**

Responsible for directing discovery and preclinical research activities related to three antibodies for the treatment of ovarian, prostate and MUC1-expressing cancers. Studies are focussed on the immune enhancing effects of antibodies and on modalities

to counteract immune regulatory networks. My group is responsible for proof-of-concept to preclinical studies in cell-based assays and animal models, mainly outsourced to CRO or in collaboration with academic institutions. Responsible for establishing internal research laboratory operations and obtaining grant funding for activities pertaining to preclinical and translational studies. Other responsibilities include development and validation of clinical immunology assays, management of clinical immune monitoring activities, establishment and coordination of research collaborations with academia and industry, participation in project teams, presentations at scientific meetings, and preparation of preclinical reports, IND submissions and BLA sections.

ALTAREX CORP., EDMONTON, AB, CANADA AND WALTHAM, MA (1996 TO 2004)

VP, Research and Director, Research and Clinical Immunology (2001 to 2004)

Responsible for directing discovery and preclinical research activities related to AltaRex's technology. Studies were focussed on the function of AltaRex antibody products to induce or inhibit immune responses (in particular T cell responses) to cancer antigens, viruses, autoimmune targets and allergens using dendritic cell systems and a variety of functional T cell assays. The lead product, OvaRex® MAb-B43.13, has advanced into Phase III clinical trials; another antibody is in Phase I. Other responsibilities included project manager for two antibody projects, management of preclinical safety studies, development and validation of bioassays and clinical immunology assay, management of immune monitoring efforts and integration with clinical team, establishment and coordination of research collaborations with academia, presentations at scientific, business development, and fund raising meetings, and preparation of preclinical reports, BLA sections and IND submissions.

Director, Preclinical Research (April 1998 to Dec. 2000)

Responsible for generation and characterization of new antibodies and constructs, biological evaluation of product candidates in animal models and *in vitro* studies, assay development and validation, preclinical testing of drug candidates for toxicology, pharmacodynamics, immunohistochemistry and pharmacokinetics.

Senior Scientist, Research and Development (1997 to March 1998)

Responsible for development of targeted photodynamic therapy. The responsibilities included project management of an immunoliposomal formulation of a photosensitizer to treat solid tumors, coordinating process development and scale-up of hypocrellin (photosensitizer) synthesis, formulation work into antibody-coated liposomes, assay development, biological evaluation (*in vitro* and *in vivo*) of various hypocrellin formulations and development of animal models. The project was sponsored by a Canadian Government grant.

Scientist, Research and Development (1996-1997)

Responsible for clone development, assay development and *in vitro* characterization of two of AltaRex's cancer vaccines (for breast and gastro-intestinal cancer).

Responsibilities included supervision of the hybridoma lab in generating new clones for cancer antigens and their characterization for specificity, epitope mapping and affinity, initial evaluation of clones for stability and productivity; testing of their therapeutic activity in various mouse tumor models, supervision of assay development for immunological assays (humoral and cellular) for research and clinical trial support, development, optimization and validation of assays for product development;

pharmacokinetic studies, and characterization of immune responses in animals and patients. Additional responsibilities included writing of SOPs, reports, publications, management of the lab.

BIOMIRA INC., EDMONTON, AB, CANADA (1994-1995)

Scientist, Research and Development (1995)

Responsible for studies on the immunological mechanisms of action of an anti-CA125 antibody for ovarian cancer. Additional responsibilities included antibody *in-vitro* characterization, assay development for immunoreactivity testing of monoclonal antibodies, for clinical immune responses and for serum quantification of the antibody in pharmacokinetic studies in patients, and analysis of serum and lymphocyte samples from clinical trials (PK and immune responses).

Postdoctoral Fellow, Research and Development (1994)

Responsible for studies on the B and T cell activation of immune complexes consisting of an antibody against ovarian cancer and the CA125 tumor-associated antigen *in vitro* and *in vivo*.

UNIVERSITY OF BONN, BONN, GERMANY (1989-1993)

Research Assistant, Clinic for Gynecology and Obstetrics (1989-1993)

Studies on antibody-coupled phthalocyanine for photodynamic therapy

Supervisor, Clinic for Nuclear Medicine (1990-1993)

Responsible for tumor marker laboratory.

PATENTS/PATENT APPLICATIONS

Method and composition for reconfirming multi-epitopic antigens to initiate an immune response (Issued), USA 6,241,985

Therapeutic Binding Agents against MUC-1 antigen and methods of their use (Issued), USA 09/641,833

Therapeutic method and composition utilizing antigen-antibody complexation and presentation by dendritic cells (Issued), USA 09/853,300

Reagents and methods for inducing an immune response to prostate specific antigen (Allowed)

Therapeutic compositions that alter the immune response (Pending)

Modulation of the immune system utilizing binding agents targeting immune regulatory receptors (Pending)

Combination Therapy for Treating Diseases (Pending)

Binding agents and their use in targeting tumor cells (Pending)

Therapeutic adjuvant (Pending)

PUBLICATIONS

ORIGINAL ARTICLES IN PEER-REVIEWED JOURNALS (SELECTED ARTICLES FROM OVER 50 PEER-REVIEWED PUBLICATIONS)

T.G. Ehlen, P.J. Hoskins, D. Miller, T.L. Whiteside C.F. Nicodemus, B.C. Schultes, K.D. Swenerton.
A Pilot Phase II Study of Oregovomab Murine Monoclonal Antibody to CA125 as an Immunotherapeutic Agent for Recurrent Ovarian Cancer. *Int. J. Gynecol. Cancer* 2004 (in press).

- J.S. de Bono, S.Y. Rha, J. Stephenson, B.C. Schultes, P. Monroe, G.S. Eckhardt, L.A. Hammond, T.L. Whiteside, C.F. Nicodemus, J.M. Cermak, E.K. Rowinsky, A.W. Tolcher. Phase I trial of a murine antibody to MUC1 in patients with metastatic cancer: evidence for the activation of humoral and cellular anti-tumor immunity. *Annals Oncol.* 15:1825-1833, 2004.
- J.S. Berek, P.T. Taylor, A. Gordon, M.J. Cunningham, N. Finkler, J. Orr, Jr., S. Rivkin, B.C. Schultes, T.L. Whiteside, C.F. Nicodemus. Randomized Placebo-Controlled Study of oregovomab for Consolidation of Clinical Remission in Patients with Advanced Ovarian Cancer. *J. Clin. Oncol.* 22:3507-3516, 2004.
- B. C. Schultes, C. N. Nicodemus. Using antibodies in tumour immunotherapy. *Expert Opin. Biol. Ther.* 4, 1265-1284, 2004.
- A.N. Gordon, B.C. Schultes, H. Gallion, R. Edwards, T.L. Whiteside, J.M. Cermak, C.F. Nicodemus. CA125- and tumor-specific T-cell responses correlate with prolonged survival in oregovomab-treated recurrent ovarian cancer patients. *Gynecol. Oncol.* 94:340-51, 2004..
- B.C. Schultes and T.L. Whiteside. Monitoring of Immune Responses to CA125 with an IFN- γ ELISPOT Assay. *J. Immunol. Methods* 279, 1-15, 2003.
- V.J. Moebs, R.P. Baum, M. Bolle, R. Kreienberg, A.A. Noujaim, B.C. Schultes, C.F. Nicodemus. Immune responses to MAb-B43.13 correlate with prolonged survival of women with recurrent ovarian cancer. *Am. J. Obstet. Gynecol.* 189, 28-36, 2003.
- J.S. Berek, B.C. Schultes, C.F. Nicodemus. Biologic and immunologic therapies for ovarian cancer. *J. Clin. Oncol.* 21, 168-174, 2003.
- J.S. Berek, O. Dorigo, B. Schultes, C.F. Nicodemus. Specific Keynote: immunological therapy for ovarian cancer. *Gynecol. Oncol.* 88, S105-109, 2003.
- C.F. Nicodemus, B.C. Schultes, B.L. Hamilton. Immunomodulation with antibodies: clinical application in ovarian cancer and other malignancies. *Expert Rev. Vaccines* 1, 35-48, 2002.
- W. Qi, B.C. Schultes, D. Liu, M. Kuzma, W. Decker, A.A. Noujaim, R. Madiyalakan. Characterization of an anti-MUC1 monoclonal antibody with potential for immunotherapy of cancer. *Hybridoma and Hybridomics* 20, 313-323, 2001.
- K.A. Berlyn, B.C. Schultes, B. Leveugle, A.A. Noujaim, R.B. Alexander, and D.L. Marm. Generation of CD4+ and CD8+ T Lymphocyte Responses by Dendritic Cells Armed with PSA/anti-PSA (antigen:antibody) Complexes. *Clin. Immunol.* 101, 276-283, 2001.
- A.A. Noujaim, B.C. Schultes, R.P. Baum, R. Madiyalakan. Antibody-Mediated Immunotherapy: Influence of Circulating Antigen on the Induction of Antigen-Specific Anti-Tumor Immune Responses. *Cancer Biotherapy&Radiopharm.*, 16, 187-203, 2001
- B.C. Schultes, C. Zhang, L.Y. Xue, A.A. Noujaim, R. Madiyalakan. Immunotherapy of Human Ovarian Carcinoma with OVAREX MAb-B43.13 in a Human-PBL-SCID/BG Mouse Model. *Hybridoma* 18, 47-55, 1999.
- B.C. Schultes, R.P. Baum, A. Niclsen, A.A. Noujaim, R. Madiyalakan. Anti-Idiotypic Induction Therapy: Anti-CA125 Antibodies (Ab3) Mediated Tumor Killing in Patients Treated with Ovarex MAb-B43.13 (Ab1). *Cancer Immunol. Immunother.* 46, 201-212, 1998.
- D. Luo, M. Geng, B. Schultes, J. Ma, D. Xu, N. Hamza, W. Qi, A.A. Noujaim, R. Madiyalakan. Expression of a Fusion Protein of scFv-Biotin Mimetic Peptide for Immunoassay. *J. Biotech.* 65, 225-228, 1998.
- B. C. Schultes, J. Reinsberg, H. Schlebusch, P. Oehr, H.J. Biersack, D. Krebs, and U. Wagner. Idiotypic Cascades in a Mouse Model treated with the Monoclonal Antibody OC125. Induction of Anti-CA 125 Antibodies after Immunization with an Anti-CA 125 (MAb OC125) Antibody by the Activation of the Idiotypic Network. *Eur. J. Clin. Chem. Clin. Biochem.* 31: 427-432, 1993.
- J. Reinsberg, B. Schultes, U. Wagner, D. Krebs. Monitoring of CA 125 in serum of ovarian cancer patients after administration of ^{131}I -F(ab') $_2$ fragments of the OC125 antibody. *J. Clin. Chem.* 39: 891-896, 1993.
- U.A. Wagner, P.F. Oehr, J. Reinsberg, S.C. Schmidt, H.W. Schlebusch, B.C. Schultes, A. Werner, G. Prietl, D. Krebs. Immunotherapy of Advanced Ovarian Carcinomas by Activation of the Idiotypic Network. *Biotechnology Therapeutics* 3: 81-89, 1992.
- B.C. Schultes, E. Fischbach, N. Dahlmann. Purification and characterization of two different thymidine-5'-triphosphate-hydrolyzing enzymes in human serum. *Biol. Chem. Hoppe-Seyler* 373, 237-247, 1992.
- S. Schmidt, B. Schultes, U. Wagner, P. Oehr, W. Decler, H. Lubaschowski, H.J. Biersack and D. Krebs. Photodynamic laser therapy of carcinomas - effects of five different photosensitizers in the colony-forming assay. *Arch. Gynecol. Obstet.* 249, 9-14, 1991.

- B.C. Schultes** and N. Dahlmann. Homogeneous preparation of human thymidine-5'-triphosphatase by electroelution from SDS/PAGE with subsequent renaturation. *Eur. J. Biochem.* 192, 201-205, 1990.

ABSTRACTS, PRESENTATIONS, POSTERS AT SCIENTIFIC MEETINGS (2000 – PRESENT)

- B.C. Schultes, H. Eng, K. Agopsowicz, C.F. Nicodemus.**
Anti-MUC1 Antibody Enhanced Helper and Cytolytic T Cell Responses with Human Dendritic Cells Presenting MUC1 Antigen or MUC1-Positive Tumor Cells. *Proc. American Association for Cancer Research* 46, 623, 2005; abstract #2649 (oral presentation).
- P.T. Taylor Jr., J.S. Berek, B.C. Schultes, D.M. Haverstick, C.F. Nicodemus.**
Utilization of CA125 Measurements in Oregovomab-Treated Patients. 36th Annual Meeting of the Society for Gynecologic Oncology, March 19-23, 2005, Miami Beach, FL (poster).
- B.C. Schultes, H. Eng, K. Agopsowicz, C.F. Nicodemus.** Potent Helper and Cytolytic T Cell Responses by Dendritic Cells Armed with MUC1-anti-MUC1 Immune Complexes. 12th International Congress of Immunology & 4th Annual Conference of FOCIS, July 18-23, 2004, Montreal, QC, Canada (poster).
- B.C. Schultes, H. Eng, K. Agopsowicz, M. Kuzma, D.L. Mann, T. Whiteside, A.A. Noujaim, C.F. Nicodemus.** Antibodies as Vaccines: Immune Complexes Facilitate Tumor Antigen Processing on MHC Class I and II, Induce Dendritic Cell Maturation and Allow for Helper and Cytolytic T Cell Activation. 21st International Conference: Advances in the Application of Monoclonal Antibodies in Clinical Oncology, June 28-30, 2004, Cape Sounio, Greece (oral presentation).
- C. F. Nicodemus, P. Taylor, B. Schultes, J. Balser, J. S. Berek.** Relationship of time to relapse (TTR) and survival post relapse (SPR): exploration of risk factors from the first annual follow-up data set of randomized pbo-controlled study of oregovomab (OV) as a consolidation therapy of patients with advanced ovarian cancer (OC). ASCO, June 5-8, 2004, New Orleans, LA (poster).
- J.S. Berek, P. Taylor, A. Gordon, B. Schultes, T. Whiteside, C. Nicodemus.** First Follow-up to Randomized Study of OvaRex® MAb (OV) for Consolidation of Clinical Remission in Pts with Ovarian Cancer (OC): Prolonged Disease-Free Survival (DFS) in Optimal Chemosensitive Pts. 35th Annual Meeting of the Society for Gynecologic Gynecology, Feb. 7-11, 2004, San Diego, CA (oral presentation).
- B. Schultes, A.N. Gordon, C.F. Nicodemus, T.L. Whiteside.** Feasibility of combined OvaRex® immunotherapy and chemotherapy in recurrent ovarian cancer. AACR Annual Meeting, July 11-14, 2003, Washington, DC, *Proc. American Association for Cancer Research* 44 (poster).
- J.L. Levin, J. Kavanagh, C. Nicodemus, B. Schultes, E. Hansen, M. Method.** Immunology and pharmacokinetic comparability profiles of OvaRex® (MAb-B43.13) in women with ovarian cancer. AACR Annual Meeting, July 11-14, 2003, Washington, DC, *Proc. American Association for Cancer Research* 44 (poster).
- J.S. Berek, P. Taylor, A. Gordon, B. Schultes, T. Whiteside, C. Nicodemus.** Randomized Pbo-controlled study of OvaRex® MAb (OV) for consolidation of clinical remission in pts with ovarian cancer (OC): prolonged disease-free survival (DFS) in optimal chemosensitive pts. ASCO, May 31 - June 3, 2003, Chicago IL *Proc. ASCO* 22 (oral presentation).
- B.C. Schultes, M.L. Kuzma, C.C. Zarozinski, K. Agopsowicz, H. Eng.** Uptake and processing of antigen-antibody-complexes by human dendritic cells: involvement of multiple receptors and in particular the mannose receptor. AAI Annual Meeting, May 6-10, 2003, Denver, CO (oral presentation).
- B.C. Schultes.** Antibodies to modulate tumor immunity. Cancer Drug Development, SMi, March 10-11, 2003, London, UK (oral presentation).
- H. Eng, M.L. Kuzma, K. Agopsowicz, C.C. Zarozinski, B.C. Schultes.** Complexation with Murine MAb-B43.13 Alters the Endocytic Trafficking of the Mucinous Tumor Antigen CA125 after Uptake by Human Dendritic Cells. Keystone Symposium "Dendritic Cells: Interfaces with Immunobiology and Medicine", March 3-8, 2003, Keystone, CO (oral presentation).
- K. Agopsowicz, M. Kuzma, H. Eng, C.C. Zarozinski, B.C. Schultes.** Opsonization with specific antibodies enhances dendritic cell presentation of apoptotic tumor cells and induction of CTL. Keystone Symposium "Cell Biology of the Immune Response", March 5-10, 2003, Keystone, CO (oral presentation).
- A. Gordon, A. Stringer, H. Gallion, T.L. Whiteside, B.C. Schultes, C.F. Nicodemus.** Induction of CA125- and tumor-specific IFN- γ T cell responses correlates with prolonged survival in patients

- with recurrent epithelial ovarian cancer treated with OvaRex® Mab-B43.13 and chemotherapy. 34th Annual Meeting of the Society for Gynecologic Gynecology, Jan. 31-Feb. 4, 2003, New Orleans, LA (oral presentation).
- B.C. Schultes, C.F. Nicodemus, J.S. Berek, T.A. Ehlen, A.N. Gordon, T.L. Whiteside.** Use of OvaRex® MAb-B43.13 as an Immunotherapeutic Treatment of Epithelial Ovarian Cancer: Experience as Single Agent post First-Line Therapy and in Combination with Chemotherapy in Recurrent Disease. 14th EORTC-NCI-AACR Symposium on Molecular Targets and Cancer Therapeutics, November 19-22, 2002, Frankfurt, Germany (poster).
- H. Eng, M. Kuzma, K. Agopsowicz, C. Zarozinski, B.C. Schultes.** Opsonization with specific antibodies enhances dendritic cell presentation of apoptotic tumor cells and induction of CTL. 17th Annual Meeting of the Society of Biologic Therapy, Nov. 7-10, 2002, San Diego, CA (poster).
- C.F. Nicodemus, B.C. Schultes, A.A. Noujaim.** Antibody Stimulated Tumor Immunity: OvaRex® MAb-B43.13 in the Treatment of Epithelial Ovarian Cancer. 4th Biennial Ovarian Cancer Research Symposium, Seattle, Sept. 19-20, 2002 (oral presentation).
- B.C. Schultes, A. Gordon, C.F. Nicodemus, R. Edwards, K. Agopsowicz, T.L. Whiteside.** Induction of tumor- and CA125- specific IFN-gamma ELISPOT responses in ovarian cancer patients treated with oregovomab correlate with improved time to progression and survival. 30th Meeting of the International Society of Oncodevelopmental Biology and Medicine (ISOBM) 2002, Sept. 8-12, 2002, Boston, MA (oral presentation).
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EXHIBIT A

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Mark Peakman
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Medical knowledge is constantly changing. As new information becomes available, changes in treatment, procedures, equipment and the use of drugs become necessary. The authors and the publisher have, as far as it is possible, taken care to ensure that the information given in this text is accurate and up to date. However, readers are strongly advised to confirm that the information, especially with regard to drug usage, complies with current legislation and standards of practice.

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Immunoglobulins

- The basic molecule is composed of two light and two heavy chains connected by interchain disulphide bonds. Within the chains, distinctive motifs (domains) are formed by intrachain disulphide bonds.
- Many immunological molecules have similar domain structures: the immunoglobulin supergene family.
- Antibodies contain regions that are highly variable in their amino acid content in different molecules: these zones form the antigen-binding site.
- Antibodies also have relatively constant parts: on the heavy chain these hold different functional properties, such as the ability to activate complement.
- Different heavy chains give rise to different classes of immunoglobulin: IgA, IgD, IgE, IgG and IgM.

cytes is an important part of the regulation of IgE production.

IMMUNOGLOBULIN EXPRESSION

B CELL SURFACE EXPRESSION

A short extension of immunoglobulin molecules at the carboxy-terminus during synthesis enables insertion into the B lymphocyte surface membrane. Surface-expressed antibody has an important role in B lymphocyte activation, as we have already mentioned in relation to surface IgD on immature B cells. Binding of antigen to surface immunoglobulin is an early event in the B lymphocyte cell cycle. In the case of mature B lymphocytes, interaction with surface immunoglobulin of the other classes leads to internalisation of the antigen. Complex antigens may then be broken down (processed) and presented to T lymphocytes (see p. 86). Through this process, antigen-specific B lymphocytes can activate T lymphocytes, leading to a concerted immune response.

ISOTYPES, ALLOTYPES AND IDIOTYPES

Many of the studies performed to characterise immunoglobulins have been performed by raising antibodies against the immunoglobulins: here the immunoglobulin has itself become the antigen. Immunoglobulin molecules have numerous distinctive structural features, and the differences and similarities between immunoglobulins are reflected in their antigenic properties. These antigens can be divided as follows. Some are present on all of the molecules of a particular immunoglobulin class or heavy or light chain type in a species and are called isotypes. For example, the ϵ heavy chain has antigens peculiar to IgE molecules, which are present on all of the IgE molecules in the members of that species. Other parts of immunoglobulins have

genetically determined differences between individuals. These are termed allotypes and are denoted by the chain affected (e.g. Gm, Em allotypes). Finally, we have to remember that the most distinctive part of an individual immunoglobulin is the antigen-binding site seated in the hypervariable region. To take the analogy of the jelly and the jelly mould again; in three-dimensional terms, the jelly mould (i.e. antibody) is as distinctive as the jelly (i.e. antigen). Injection of a human antibody into a mouse should result in the production of some murine antibodies recognising parts of the antigen-binding site. Indeed, this is the case, and such determinants in that human antibody are termed idiotypes. Idiotypes are antigenic determinants in the antigen-binding site (the hypervariable regions, or CDRs) and as such can only be identified by raising an antibody response to them. The antigenic epitope within the antigen-binding site is termed an idioepitope, and a collection of idiotopes is the idioepitope. It appears that anti-idiotypic antibodies are a normal feature of the antibody repertoire, and it has been proposed that interaction between anti-idiotypes and the idiotypes on antibody molecules has a role in regulation of the immune response (see p. 105).

PRIMARY AND SECONDARY ANTIBODY RESPONSES

When antigen not previously encountered is injected into an animal, and the antibody response measured, several observations can be made. The antibody response is detected 5–10 days after antigen challenge, rises over the next 10–20 days, and then declines to a low level without ever completely disappearing. If the same antigen is administered again several weeks later, the antibody response is more rapid, hits a higher peak level, and declines, but to a higher baseline level than previously seen (Fig. 4.9). These two responses, therefore, differ qualitatively and quantitatively, and

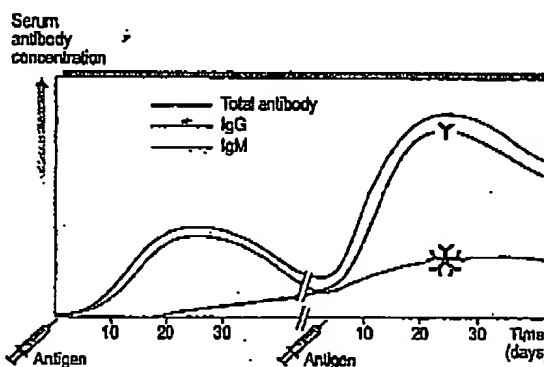


Fig. 4.9 Primary and secondary antibody responses. Administration of an antigen at day 0 is followed by a primary antibody response, comprising predominantly IgM, at about 10 days. Rechallenge with the same antigen some weeks later causes a much more rapid rise in antibody levels and to a higher peak; this time the response is predominantly IgG (secondary response).

BASIC AND CLINICAL IMMUNOLOGY

are termed primary and secondary. If a different antigen is given at the second challenge, a primary response to that antigen is seen. The secondary response is, therefore, antigen-specific, and demonstrates acquisition of memory in the immune response. There is also a higher intensity to the secondary response.

The qualitative differences between primary and secondary responses extend further than just the speed of appearance of antibody. The primary response is seen predominantly as antibody of the IgM class. IgG antibody specific for the same antigen begins to appear towards the end of the primary response. The secondary response, however, is predominantly IgG class antibody. There is another feature of the IgG in the secondary response: it has a higher affinity for antigen than antibody in the primary response. The genetic and cellular basis for switching from IgM to IgG class antibody is discussed below. The generation of high-affinity antibodies will also be discussed in greater depth, but in simplistic terms it can be viewed as the application of Darwinian evolutionary principles to B lymphocyte survival: those B cells with the highest affinity for an

antigen in short supply compete most effectively and undergo positive selection.

HAPTENS

Some molecules are too small of themselves to generate an antibody response when administered to an animal. However, if coupled to a larger protein (a carrier), these small molecules may elicit production of antibodies that are able to bind the small molecule directly, in the absence of the carrier. Such molecules are termed haptens. Much of the early work on the nature of antigen-antibody binding was carried out using hapten-carrier systems.

CLONALITY IN ANTIBODY RESPONSES

Plasma cells — the end stage of B lymphocyte differentiation — produce and secrete immunoglobulin. More specifically, a single plasma cell produces a single antibody, with one heavy chain type, one light chain type and one conformation of antigen-binding region. This

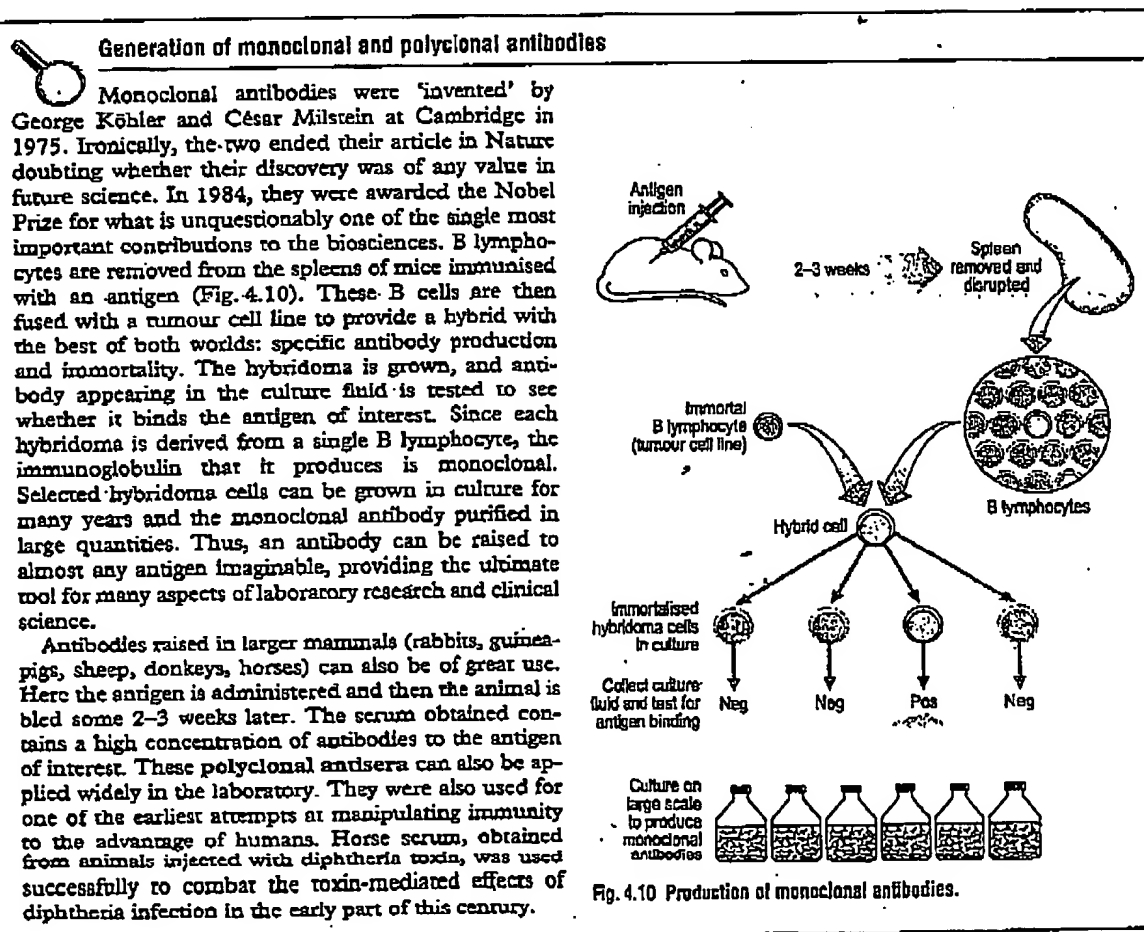


EXHIBIT B

SM: Saw "mm"
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Medical Immunology

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Physiology of the Immune System 23

the site of antigen entrance into the body. Within the lymph node, there is mitotic activity in the paracortical and medullary areas, regions of T-cell concentration. These lymphocytes divide and differentiate, becoming immunoblasts. The cells enlarge and their cytoplasm becomes packed with ribosomes. However, there is no evidence of antibody synthesis and the cortical nodules remain quiet and undisturbed.

Similar events unfold at the site of antigen administration, and there is a marked infiltration of blast cells, lymphocytes and macrophages. Thus, unlike the humoral response where the cells remain in the node and exert systemic effects by the secretion of antibody molecules, here the cells themselves migrate to wherever antigen might be. Not surprisingly, this is termed *cell-mediated immunity* and it is attributable to the T-cell population. The activated T cells participate directly in the destruction of the inciting antigen against which they are specifically sensitized. Such T cells are referred to as *T killer cells*.

The Combined Response

The separation of the immune response into a B-cell-mediated humoral immunity and a T-cell-mediated cellular immunity is somewhat misleading. Most antigens do not elicit pure B- or pure T-cell responses as we have so far described; rather both types of responses are evoked. This is the case with a commonly employed experimental antigen, the sheep erythrocyte (SRBC). Injection of this antigen leads to blast-cell activity in the T-cell paracortical areas by two days. Several days later follicular activity is noted, germinal centers are formed, and antibody is produced. Thus, the pattern of lymph node reaction is actually a combination of the two patterns described above, and both effector limbs of the immune system, humoral and cellular, are

activated to counteract the potential threat of this and most other antigens.

The Systemic Response to Antigen

Despite the best efforts of the host to localize an immune response within the local lymph nodes, this is not always possible. If the antigen is introduced via a systemic route (for example, in a blood transfusion), if the antigen is an extremely aggressive pathogen, or if the dose of antigen is too large to be contained by the local lymph nodes, antigen may then enter the circulation and spread throughout the body. When this occurs, the task of removing the antigen falls on those tissues of the RES associated with the circulatory system. These include the spleen, liver and lung. Their structure allows them to function as both filter and phagocyte for circulating antigens.

The ability of the RES to clear an antigen from the circulation is a function of several factors. Of great importance are the physical properties of the antigen. Large, particulate or colloidal molecules are readily cleared: the liver can remove 80 to 90 per cent of such antigens in a single passage. Small, soluble particles are much less easily removed. The presence of antibody directed against the antigen enhances clearance. The removal of antigen-antibody complexes from the circulation can be of great significance, since free complexes can play a nefarious role in the pathogenesis of several diseases, including serum sickness and several types of glomerulonephritis. A third factor is the ability of some antigens to nonspecifically stimulate RES activity. Prominent among these are bacterial cell wall components such as lipopolysaccharides (LPS).

A large challenge of antigen can induce proliferation of the RES and hypertrophy of the involved organ. It has been suggested that an excessively large dose of antigen can exhaust the RES, leaving the host vulnerable to secondary infection.

24 The Foundations of the Immune Response

Among the organs of the RES, the liver probably serves as the major filter for antigen. The spleen, however, is constructed much like the lymph node, and events closely analogous to the events of an immune response within a lymph node can occur there. The spleen can therefore mount a specific immune response and rapidly release antibody and sensitized cells directly into the circulation.

The Effect of Antigen on the Circulation of Lymphocytes

Not only can lymphocytes that are specifically reactive against the inciting antigen proliferate within the lymph nodes and spleen, but the large population of recirculating cells can be mobilized to the site of reactivity.

The introduction of antigen disrupts the normal pattern of lymphocyte circulation. There is a rapid disappearance of cells capable of reacting to the antigen from the recirculating pool.²⁹ They are specifically sequestered in the lymph nodes draining the site of antigen invasion. At this time, a second administration of antigen at a site distal to the original one will evoke a very poor response in the local lymph nodes. This may be because all the antigen-specific cells have been summoned to the site of the initial attack and it may take a while to reappportion these cells between the two locales.

Also serving to localize the antigen-specific lymphocytes in the lymph nodes is the nonspecific influx of granulocytes into the involved nodes. These cells plug up the lymphatics and prevent lymphocyte egress. Not all of the factors leading to the dramatic decline in the rate of lymphocyte egress from a lymph node have been defined.^{30,31}

It appears that the antigen itself does not necessarily have to be carried to a lymph node in order to incite a reaction there. It is clear that such antigen localization does occur, but recent evidence

suggests that circulating lymphocytes can contact an antigen, become sensitized to it, and then migrate to the nearest lymph node carrying a stimulatory message to the resident lymphocytes. This process has been demonstrated only for the T-cell population and is called "recruitment." The recruiting T cell does not carry antigen back to the node. The only requirement for this activity described thus far is that the T cell needs a surface-expressed protein of an, as yet, unspecified nature in order to recruit effector cells.³² Recruitment may be one way by which host sensitization and eventual rejection of a renal transplant takes place. The release of donor-tissue antigens directly into the blood is probably important as well (see Chap. 9).

Interdependence of Humoral and Cellular Responses

Thus far we have presented a very simple picture of an immune system neatly divided into two parts, a cellular limb and a humoral limb. Work of the past decade, however, has forced a revision of this complicated view. Essential to attaining new sophistication in our understanding of the immune response has been the observation that the ability to mount humoral response to most antigens requires the concomitant activity of T cells. Such T-cell activity is called *T helper activity*, and the cells involved are called *helper cells* (Fig. 1-21).³³ Antigens that demand T-cell cooperation in eliciting B-cell humoral response are called *T-cell-dependent antigens*. There are some exceptions, and antigens which seem to require only B-cell activity in order to induce a humoral response are called *T-cell-independent antigens*. *Helper cells* do not make antibody. Rather, their role is to interact with the B population so that the latter can mount an effective humoral response. How this is accomplished will be discussed in Chapter 5.

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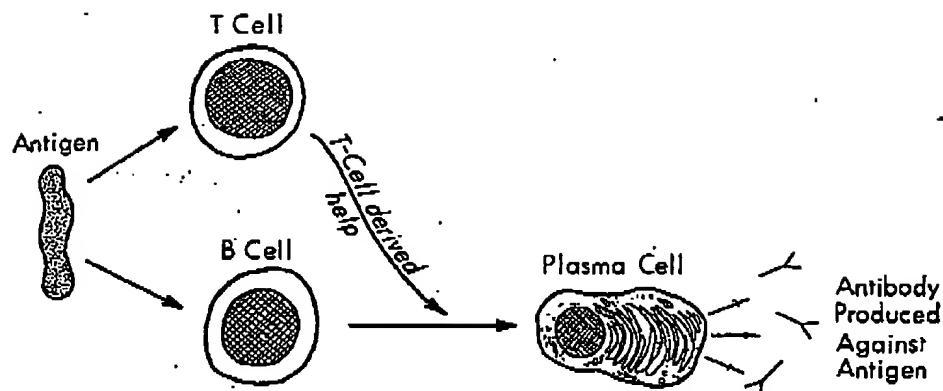


Fig. 1-21. T helper cells are required for the production of a B-cell-mediated humoral response to many antigens.

The existence of T helper cells explains the observation we alluded to earlier, namely, that neonatal thymectomy not only compromises T-cell function but also significantly reduces the B-cell humoral response against most antigens.³⁴ Thymectomy removes T cells involved in both effector and helper functions.

Are these T helper cells a distinct subpopulation from the T killer cells, or can one cell subserve both functions? The answer to this is not clear at present, but most evidence indicates that two separate T-cell subpopulations are involved (see Chap. 5).

The Secondary Response and Immunologic Memory

Up to this point we have investigated the immune response to antigens that the immune system has never seen before. The responses we have described are therefore called *primary* and differ significantly from the body's response to a second exposure to an antigen. A *secondary* response is more rapid and of greater intensity than a primary response. This observation illustrates another basic characteristic of the immune system—*memory*. Secondary responses are called

anamnestic because the immune system "remembers" having encountered the antigen before. The first encounter has primed the host so that upon subsequent exposure the immune system is effectively alerted and prepared to deliver a devastating blow against the antigen.³⁵

Immunologic memory is a characteristic of both B and T cells. The responsibility for anamnestic responses is felt to reside in specialized B and T lymphocytes called memory cells, generated during initial exposure to antigen.

The secondary response in the lymph nodes and the spleen appears much like the primary response, but events happen more quickly and the degree of cellular activity is greater.³⁶ Antigen penetrates rapidly into the cortical follicles where it is rapidly bound to the surface of reticulum cells and macrophages. This rapid immune adherence of the secondary response is due to the presence of antibody molecules that can bind to the surface of these cells and hence are termed *cytophilic*. These cytophilic antibodies exist as the result of the previous primary response. Upon second introduction of antigen into the lymph node, the antigen can combine with these antibody molecules and the resulting complexes

EXHIBIT C

KINETIC STUDY OF T LYMPHOCYTES AFTER SENSITIZATION AGAINST SOLUBLE ANTIGEN

I. Separation on Density Gradients¹

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Sensitized T cells in inguinal lymph nodes (LN) of rats immunized with ovalbumin and adjuvant can be separated on BSA density gradients from T cells responding to Con A and PHA. Further, these OA-sensitized T cells are separable from B memory cells responsive to OA. On days 9 and 17 after sensitization, OA-reactive cells peak and are found in enriched numbers in the lighter bands A + B (B-band), and C, respectively, whereas Con A and PHA reactive cells, on both days, are concentrated in bands C and D, respectively. On day 9 at least 20 to 30% of B-band cells can reinstate DNA synthesis and produce high concentrations of lymphotoxin in response to OA in culture, but show only a marginal blastogenic response to Con A or PHA and cannot produce lymphotoxin in response to PHA. These cells are defined as OA-sensitized T cells because a) treatment with rat isoantiserum specific for T lymphocytes plus complement abolished their ability to incorporate ³H-thymidine in response to OA and Con A or PHA; b) membrane-bound immunoglobulin could not be detected with indirect immunofluorescence staining; c) histologic studies demonstrate massive hypertrophy of thymus-dependent paracortical areas and absence of germinal centers in inguinal LN at this time, and d) they produce lymphotoxin after challenge with OA, but not with PHA, whereas cells of the other bands produce lymphotoxin in response to PHA, but not to OA. Addition of purified macrophages to the various cultures did not affect the levels of response observed. Day 17 C-band cells are regarded as B-memory cells because treatment with T-specific antiserum plus complement enhanced their response to OA, many germinal centers now are present in inguinal LN, and a significant proportion of cells of bands B and C, negative on day 9, show positive immunofluorescence staining at this time.

A fundamental question concerning the sensitized T lymphocyte is whether it differs qualitatively, as well as quantitatively, from its so-called immunocompetent precursor which has not previously encountered antigen. In attempting to answer this question, the identification of T cells sensitized by exposure to membrane (histocompatibility, red cell, tumor, or microbial) antigens has been accomplished by several laboratories (1-9). These cells have been described as nonadherent (4, 9) intensely pyroninophilic (4, 10, 11) small to medium-sized

lymphocytes (12), with slightly more voluminous cytoplasm than other small lymphocytes (1, 9, 13, 14), and possessing a uropod (3, 9). They are relatively short-lived and said to possess ultrastructural characteristics akin to cells activated by mitogens (3). They tend to localize at nonspecific inflammatory sites (14-16). They are found in low to moderate density subpopulations when separated on density gradients (5).

Our purpose was to isolate T memory cells sensitized by soluble protein antigen, as a concentrated subpopulation, separate and distinct from B memory cells responsive to the same antigen. We approached the problem on the premise that, after immunologic encounter, T cells undergo progressive differentiation and maturation, correlated with changes in cell size (1, 13, 17). With a convenient cell separation technique based on density, this study permitted us to recover ovalbumin-sensitized T cells differing fundamentally in size as well as in other properties from their unsensitized counterparts.

MATERIALS AND METHODS

Animals and immunizations. Inbred DA rats (males, 6 to 8 weeks of age) were immunized by injection of OA³ (ovalbumin, 5 × crystalline, Nutritional Biochemicals Corp., Cleveland, Ohio): 100 µg in Freund's complete adjuvant (CFA) delivered by multiple injections into both hind footpads (18). For each kinetic, immunofluorescence, and adherent cell study, animals to be compared were immunized with a single batch of antigen in CFA prepared on the same day.

Cell suspensions. Inguinal lymph nodes were used as a source of sensitized lymph node cells (LNC). Cells were washed once in HBSS (Hanks' balanced salt solution, Grand Island Biological Co., Grand Island, N. Y.) and resuspended for counting in a hemocytometer. Cell viability was determined with a 0.2% solution of trypan blue (Allied Chemical Corp., New York) in HBSS; cells unable to exclude the dye were counted as dead.

Bovine serum albumin (BSA) gradient separation. At various intervals after sensitization, inguinal LNC were separated by differential flotation on discontinuous albumin gradients. BSA (Lot 126, pH 6.9, 300 milliosmoles, Miles Laboratories, Kankakee, Ill.) was diluted with RPMI-1640 (Grand Island Biological Co.) on a v/v basis. Five milliliters of 30% BSA solution were carefully overlaid with 7 ml each of 27, 24 and 20% BSA. Cells resuspended in 5 ml 10% BSA were gently overlaid on the 20% BSA layer. Centrifugation was carried out at 12,500 rpm for 30 min at 34°F in a Beckman Spinco SW 25.1

³ Abbreviations used in this paper: A, B, C, D and F: bands (and pellet) respectively, obtained on density gradients; BSA, bovine serum albumin; CFA, complete Freund's adjuvant; OA, crystalline hen ovalbumin; Con A, concanavalin A; PHA, phytohemagglutinin; LNC, lymph node cells; HBSS, Hanks' balanced salt solution.

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² Postdoctoral Fellow, Arthritis Foundation.

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rotor (Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.). Discrete bands of cells which formed at the density interfaces were harvested with an 18-gauge needle and 15-ml syringe from the top of the tube. They were designated A, B, C, and D, beginning with the top band (least dense cells) and P (pellet). The total numbers of cells present in both inguinal lymph nodes of 12 rats were obtained immediately after the cells were teased into suspension. Viability assessed by trypan blue dye exclusion was > 90 to 95%. In order to obtain accurate total yield figures after separation on density gradients, the cells associated with the various bands were counted in their respective BSA solutions, before washing. (The interband regions were collected with the cells of the appropriate band.) The cells were then washed three times in large volumes (cell losses are minimized when BSA solutions are made very dilute) to ensure removal of the BSA. After the final wash, they were re-counted and their viability assessed as > 95%, excluding P, which was > 85%. Because of low cell yields, bands A and B were combined throughout the experiments reported here and are collectively referred to as Band B.

Macrophage purification. Purified macrophages obtained from peritoneal cells of normal donors were prepared as described previously (19). This cell preparation contained > 98% macrophages, as judged by neutral red staining, with a viability of > 90%.

Cytotoxicity assay. The T-specific antiserum used in these studies was a gift of Dr. David Lubaroff and prepared as described previously (20). DA inguinal LNC were incubated with Black Hooded anti-Lewis thymus serum, normal rat serum, or RPMI/1% BSA at 4°C for 45 min. Each tube contained 1×10^7 cells in 1 ml RPMI plus 1 ml antiserum (or normal serum). The cells were then washed and 1×10^6 cells/ml were incubated for another 45 min with 1 ml complement at 37°C (source: pooled frozen guinea pig serum diluted 1:2, previously absorbed with agarose to eliminate natural cytotoxic antibody). The proportion of cells killed was assessed by trypan blue dye exclusion (200 cells of each type were counted) and quantitated as follows:

Cytotoxic index =

$$\frac{\% \text{ dead with antiserum minus } \% \text{ dead with normal serum}}{100 - \% \text{ dead with normal serum}}$$

The cells were resuspended in 1.0 ml medium and layered on the top of a two-step BSA density gradient consisting of a bottom layer of 35% BSA overlaid with 10% BSA. The gradient was then centrifuged and the viable cells (99% viable-trypan blue exclusion) were removed from the 10/35% interface. The cells were cultured as described below.

Immunofluorescence assay. Indirect immunofluorescence assays were performed on viable cell populations by methods described previously (21). Washed cells, 5×10^4 , were incubated with 0.1 ml polyvalent rabbit anti-rat immunoglobulin (Microbiological Associates, Bethesda, Md.) in small tubes, for 15 min in the cold. The cells were then washed three times in HBSS without serum and incubated with 0.1 ml fluorescein-conjugated goat anti-rabbit immunoglobulin (Microbiological Associates, Inc.) in the cold for 15 min. The cells were again washed three times and examined with a Zeiss fluorescence microscope under both dark field and UV illumination. Only healthy living cells (those not showing cytoplasmic staining) were counted. For each suspension, at least 200 living cells were counted to obtain a figure for the percentage of fluorescent cells.

Lymphotoxin assay. The detailed procedure has been described elsewhere (18). Briefly, 1×10^7 viable inguinal LNC from different fractions separated on BSA gradients or unfractionated sensitized or normal LNC, together with either 25 $\mu\text{g/ml}$ OA or 1.4 $\mu\text{g/ml}$ phytohemagglutinin (PHA), were added directly to flasks containing mouse A9 fibroblast monolayers (prepared 1 day earlier by seeding of 4×10^5 A9 cells) in 4 ml of complete medium (Eagle's minimum essential, 10% fetal calf serum, 1% L-glutamine, 100 units/ml each penicillin-streptomycin). At 72 hr after addition of cells plus OA or PHA, dead cells were removed by gentle washing and the surviving fibroblasts were removed from the flask wall by incubation with 0.25% vickase solution. The surviving cells were collected and enumerated in a Coulter counter. Cytotoxicity was calculated as follows: % cytotoxicity = $100 - (A/B \times 100)$, where A = surviving fibroblasts in the presence of sensitized LNC plus OA or PHA and B = surviving fibroblasts in the presence of normal LNC plus OA or PHA. In some experiments, 1 to 4% purified peritoneal macrophages were added to the various cultures. Dose-response titrations were performed for cells of each band with each stimulant to ensure that the responses reported were maximal.

Lymphocyte transformation. Cells were cultured in microtiter plates (22), each well receiving 5×10^5 unfractionated inguinal LNC, or 5×10^4 viable cells from subpopulations obtained by density gradient ultracentrifugation, in 0.025 ml RPMI plus penicillin-streptomycin 100 units/ml each, 0.025 ml RPMI plus 24% heat-inactivated normal DA serum, and 0.025 ml of OA 100 $\mu\text{g/ml}$, PHA 1.1 to 1.4 $\mu\text{g/ml}$, concanavalin A (Con A) 5 $\mu\text{g/ml}$ (mitogens: Difco Laboratories, Inc., Detroit, Mich.), or medium (control). In some experiments, 1 to 4% normal purified peritoneal macrophages were added to the cultures. Dose-response titrations were performed for cells of each band with each stimulant to ensure that the responses reported were maximal. Cultures were incubated for 72 hr and pulsed with ^3H -thymidine during the final 24 hr. Results are expressed as mean TCA-precipitable counts per minute (cpm) of duplicate experimental cultures minus background (B-C).

Quantitation of OA-sensitized cells. Cells were cultures as above. However, in this instance ^3H -thymidine was added at 6 hr and the cells were harvested at 24 hr after the start of culture. In order to obtain accurate total counts of cells harvested, care was taken to scrape out each well with a rubber policeman and to retain the residual cells removed from the well and DNase was added to the cell suspensions to free any clumps. The cells were washed and smears were prepared for each fraction and stained with methyl green pyronin. Two hundred cells from each fraction were counted. Only cells measuring > 12 μ in diameter with pyroninophilic cytoplasm were counted as blasts. Comparable cultures representing the various fractions were processed for scintillation counting.

Histology of inguinal lymph nodes. At various times after footpad injection of OA in CFA, both inguinal lymph nodes were removed for histologic examination. Inguinal lymph nodes were also removed from normal animals. Histologic studies were performed on paraffin sections prepared from tissue fixed in Carnoy's solution and stained with hematoxylin and eosin. At least two sections from each lymph node of a given animal were evaluated.

RESULTS

Changing cell yields with time after immunization. The

fractionation technique used throughout these experiments resulted in a loss of cells amounting to 40 to 60% of the total placed on the gradients. It was assumed for purposes of calculation that the losses during banding and washing were nonselective.

The numbers of cells in both inguinal lymph nodes from 12 rats are given in Figure 1. Total cell yields after immunization increased rapidly, after a lag of 2 to 3 days, reaching a peak 5 times control values at 9 to 12 days, then declined slowly to 3 times control at 30 days. The rise reflected successive increases first in cells of band B on day 5, followed by C, then D. The numbers of cells of band B reached a peak value four times control at 9 days, after which they declined rapidly to 1.5 times control at 14 days. These cells again increased in numbers at day 17, after which they declined to control levels after day 21. Cells of band C also reached peak values at day 9, then declined, to increase again and reach a second peak approximately 2.5 times control at day 17. The C and B bands made up one-third to one-half the total at 9 days, but only 20% of the total at 30 days. The rise in cell numbers in band D occurred as those of bands C and B fell. Cells of band D reached peak values at day 14, when they were 3.5 times control, then fell slowly as cell numbers of the pellet rose. With the exception of band B, the cell numbers retrieved from the various bands and from the unfractionated LN at 30 days were well above control levels.

Histologic observations. Normal inguinal lymph node sections showed many well-defined follicular B areas. Only a few germinal centers were seen. The paracortical thymus-dependent areas occupied approximately 60% of the total lymphoid

area observed in each section. By day 5 after sensitization, cells of the thymus-dependent areas proliferated to such an extent so as to comprise approximately 70% of the total lymph node area observed. Follicles still were readily discernible, occupying approximately 30% of the total area and germinal centers were rarely seen. By day 9, cells of the thymus dependent area occupied approximately 90% of the total lymph node area and caused crowding out of the follicles which now could hardly be recognized. No germinal centers were seen. By day 17, the intense hypertrophy of the thymus-dependent area had subsided; it occupied approximately 40 to 50% of the total lymph node area studied and resembled that of a normal lymph node. Follicles regained their normal architecture and accounted for about 60% of the total area in each section. Many germinal centers (more than 15 per section) were now present.

Changing reactivity of LNC to antigen with time after immunization. Unfractionated LNC giving a response to OA in culture first appeared at day 5, peaked at 9 to 12 days, then disappeared abruptly (Fig. 2). A second rise in cells reactive to antigen was observed at 17 days. When the cells were separated by density gradient ultracentrifugation, antigen-reactive cells, at day 5, were found only in band D. At day 7, they were present in C and D, and in the pellet. At day 9, responsiveness to antigen was observed in all bands, but the magnitude of the response to antigen was greatest in the cells of B. At day 12, cells of bands B, C, and D declined in their ability to respond to antigen, and by 14 days, only those in D remained responsive. A second rise in cells reactive to antigen was observed on day 17, almost entirely in band C. At day 21, and thereafter, no further response to antigen was observed in cells of any band. Cells of the pellet gave no response to antigen throughout this study, except for low values on days 7 and 9.

When day 9 B-band cells were stimulated *in vitro* with other antigens (bovine γ -globulin, BSA), no proliferative responses were observed.

Reactivity of LNC to PHA and Con A after immunization. PHA reactivity was found principally in cells of band D throughout this study (Fig. 3). There was a slight decrease in PHA reactivity in band C on day 5 and thereafter, perhaps, corresponding to the increased proportion of antigen-reactive cells in this fraction. Cells of band B showed low and constant PHA reactivity throughout, varying by less than 100 cpm. Cells of P gave a similar low response; this fell to very low levels at 17 and 21 days.

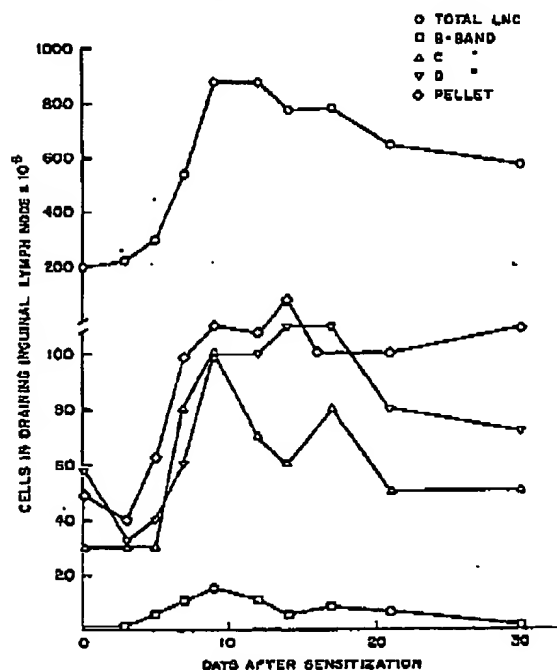


Figure 1. Changing cell yields in inguinal lymph nodes with time after immunization. DA rats sensitized by footpad injection of 100 μ g OA in complete adjuvant, O—O, cells harvested from both inguinal lymph nodes of 12 rats counted before banding. Cells separated on 5-step BSA gradients (10/20/24/27/30%). A + B = least dense, combined because of low yields. Bands counted before washing.

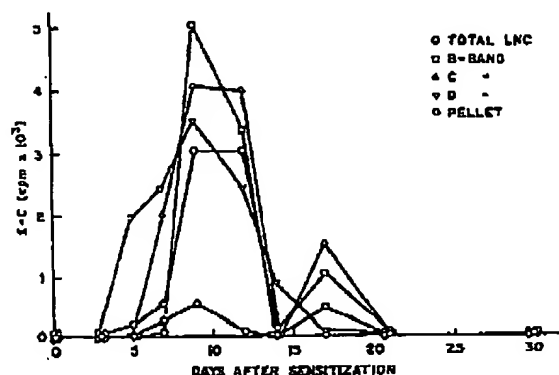


Figure 2. Changing reactivity to antigen of sensitized LNC separated on BSA gradients. Values are given for cultures of 5×10^5 cells, expressed cpm S-C (C: range 911-2792).

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In another series of experiments, the response of sensitized LNC to Con A was studied. The peak response to Con A was in band C, rather than in band D throughout (Table I), with the result that PHA to Con A ratios were strikingly different in these two bands.

Effect of addition of macrophages to cultures. By the neutral red technique, no more than 1 or 2% of the cells from the different bands could be classified as macrophages. However, when 2% unstimulated purified peritoneal macrophages were added to cultures of cells from each band in an attempt to potentiate their proliferative responses to OA, or to PHA or Con A, no potentiation was observed. It is possible that the crowded conditions of the microculture system obviate the need for additional macrophages (23).

Separation of antigen from PHA and Con A reactivity in sensitized LNC. Closer analysis of these data shows a time-dependent dissociation of the reactivity to antigen and PHA (Fig. 4). On day 9, cells of band B were highly reactive to antigen and almost unresponsive to PHA. In contrast, the cells of D reacted well to PHA and showed little reactivity to antigen. This was true for each of four experiments shown. The dissociation of reactivities was much less clear-cut at 5 days.

A second series of experiments showed dissociation of the reactivity to antigen and Con A (Table I). The peak response to Con A was always highest in band C. Cells of band B, again, were highly responsive to OA and almost unresponsive to PHA, and they responded poorly to Con A. When OA and PHA, or

OA and Con A were added simultaneously to the same culture, a summation of counts was observed (data not given).

Total responses of LNC after immunization. Total responses to OA and PHA for the inguinal lymph nodes of a single rat were estimated by multiplying the counts per minute responses obtained with 5×10^5 cells of a given band in culture by the estimated number of cells of that fraction in the node (Fig. 5). Two points stand out: Although the relative responsiveness to antigen was greatest in band B at day 9, the largest total response to antigen actually was seen in bands C and D. Secondly, the total response to PHA also strikingly increased after sensitization. At 9 days and thereafter, it was more than double that at 5 days, although the response to antigen declined considerably during this period.

Characterization of reactive LNC subpopulations on days 9 and 17. 1. When cells of band B at day 9 were treated with antiserum directed against an antigenic marker of rat thymocytes and peripheral T cells and not cytotoxic for immunoglobulin-bearing cells (18) plus complement, the cells surviving such treatment were virtually unresponsive to OA, PHA, and Con A in culture (Table II). In contrast, when cells of band C on day 17 were treated with this antiserum, and the surviving cells subsequently cultured, the proliferative response to antigen was more than twice that of untreated cells, while the proliferative responses to PHA and Con A again were obliterated.

2. Indirect immunofluorescence staining of cells for mem-

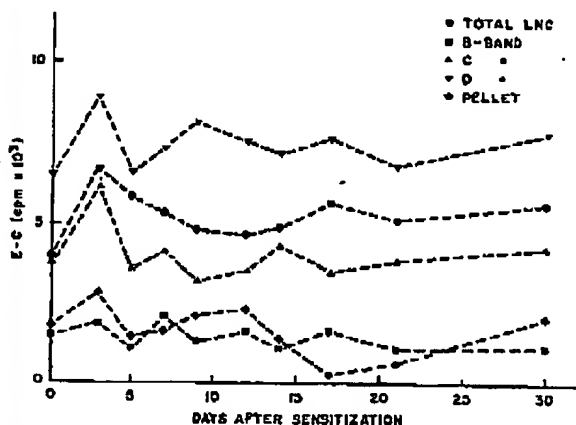


Figure 3. Reactivity to PHA of sensitized LNC separated on BSA gradients. Values are given for cultures of 5×10^5 cells, expressed as cpm E-C (C: range 811-2792).

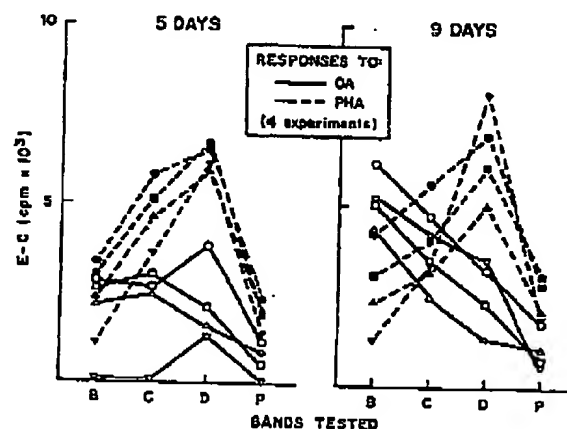


Figure 4. ^3H -thymidine incorporation by cells of the different bands obtained at 5 and 9 days, when exposed to OA and PHA. Values as in Figures 2 and 3.

TABLE I

Comparative ^3H -thymidine incorporation by sensitized LNC separated on BSA Gradients after stimulation with PHA or Con A*

Band	Days after Sensitization											
	Day 5				Day 9				Day 17			
	OA	PHA	Con A	PHA/Con A Ratio	OA	PHA	Con A	PHA/Con A Ratio	OA	PHA	Con A	PHA/Con A Ratio
A + B	147	1248	1997	0.63	6081	842	2032	0.40	<1	740	1834	0.40
C	364	5010	7318	0.69	3670	5491	8016	0.70	2017	3214	5742	0.56
D	1364	6720	1900	3.54	2659	8243	6030	1.37	1394	7897	8009	2.62
Pellet	<1	1561	866	1.83	1238	661	857	0.77	640	168	1151	0.15
Unfract. LNC	418	4860	7142	0.68	2666	6060	5948	1.02	738	5307	3992	1.33

* cpm E-C (C: range 1899-2507).

Figure 5. Total response to OA and PHA for both inguinal lymph nodes of a single DA rat estimated by multiplying the cpm responses obtained with 5×10^4 cells of a given band in culture by estimated numbers of cells of that fraction in the nodes (calculated from yield figures given in Figure 1).

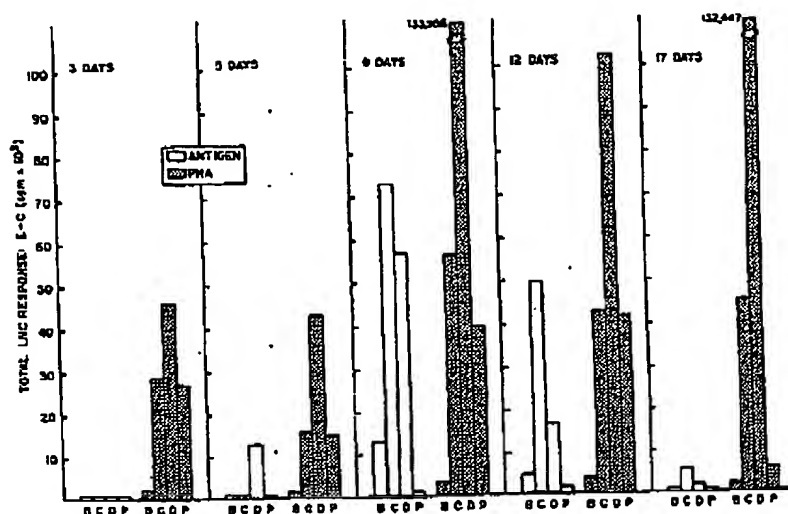


TABLE II

³H-thymidine incorporation after treatment with T cell-specific antiserum and complement^a

Stimulant	Day 9 (Untreated)	B-Band (Antiserum)	Day 17 (Untreated)	C-Band (Antiserum)
OA	4297	225	4501	9760
PHA	1274	150	3501	181
Con A	1561	221	4927	325

Cytotoxic Index: 56

Cytotoxic Index: 35

^a cpm E-C (C: range 2016-2217).

TABLE III

Percentage of OA and PHA responsive LNC in subpopulations obtained on day 9 following sensitization

Band	Cells Surviving at 24 hr ^a	OA Responsive Cells ^b	³ H-T Uptake at 24 hr
	$\times 10^4$	%	cpm
A + B	4.8	20.1	1670
C	4.5	8.1	629
D	4.9	3.4	420
Pellet	3.4	1.4	122
Unfract. LNC	4.9	5.4	532

		PHA Responsive Cells	
A + B	4.4	0.9	227
C	5.0	9.0	729
D	5.0	27.0	1710
Pellet	2.4	2.4	177
Unfract. LNC	5.0	13.5	1119

^a 5×10^4 cells cultured. Counts of cells at 24 hr after DNase treatment to eliminate clumping.

^b In 6×10^4 cells originally cultured with OA or PHA less percentage blasts in controls. Only cells $> 12 \mu$ in diameter, with pyroninophilic cytoplasm were considered to be blasts.

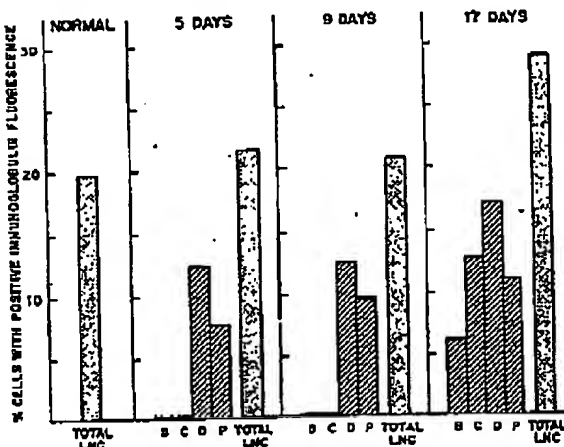


Figure 6. Percentage of inguinal LNC bearing membrane-bound immunoglobulin at various days following sensitization.

brane-bound immunoglobulin was also carried out. On days 5 and 9, both bands B and C were entirely negative (Fig. 6). In contrast, during the increase of antigen-reactivity observed at 17 days, these bands included 6 and 12% immunoglobulin-positive cells and there was a significant increase in the proportion of positive cells in the LNC population as a whole (to 28%).

Quantitation of OA and PHA responsive cells at 9 days. At least 20% of the cells present in band B at day 9 are capable of mounting a blastogenic response to OA (Table III). Actually this number may be greater since only cells measuring $> 12 \mu$

in diameter and showing pyroninophilic cytoplasm were counted and there may be smaller responding cells which were not included in the count. In contrast, only 3.4% of the cells of band D responded to OA by blastogenesis whereas 27% responded to PHA. PHA-responsive cells amounted only to 0.9% of band B. The numbers of OA-responsive cells in C-band and in unfractionated LNC samples were much lower than in band B and those in the pellet were negligible. With PHA as stimulant, values for C-band and unfractionated LNC samples were much lower than in band D. Again, the numbers in the pellet were negligible. If the numbers of blasts present in band B are calculated on the basis of cell diameter of 10μ , instead of the more rigid criterion of 12μ , the percentage of cells capable of mounting a blastogenic response to OA increased to 30%, whereas the numbers of PHA-responsive cells increased only to 4%. The only DNA synthesis observed at 24 hr was by cells of band B stimulated with OA and of band D when PHA was the stimulant. Unfractionated LNC samples also reacted to PHA.

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To determine whether the blasts in the B-band at day 9 actually were OA-responsive cells rather than cells responding to blastogenic factors (24, 25) released by the OA-responsive cells, a mixing experiment was performed. 2.5×10^4 B-band cells from rats stimulated 9 days previously with bovine γ globulin in CFA were added to 2.5×10^4 B-band cells from OA-sensitized animals. Five $\times 10^3$ cells representing the two populations were cultured with OA, collected at 24 hr, and smears were evaluated. In this case, the numbers of blasts dropped to 8% of the total cells in band B. There was no change in the numbers of blasts when PHA was the stimulant.

Lymphotoxin production and other properties of separated subpopulations. The relative production of lymphotoxin by cells of the various bands stimulated with OA or to PHA on different days after sensitization will be reported elsewhere (26). Briefly, sensitized cells of band B at 9 days, on restimulation with OA *in vitro*, showed great capacity to kill a target monolayer of fibroblasts (> 90%) and we have demonstrated that they do so by producing lymphotoxin (21). They did not do so when stimulated with PHA, with or without the addition of unstimulated purified peritoneal macrophages. In contrast, cells of band D and the pellet produced no killing when exposed to OA at this time, but killed > 90% of fibroblasts when PHA was the stimulant. Cells of band C were cytotoxic with both OA and PHA at day 9, as were unfractionated LNC. By day 17, no effect was produced with OA by cells of any individual band, and virtually none by the unfractionated LNC.

DISCUSSION

Although the technique used to fractionate inguinal LNC resulted in cell losses of 40 to 60%, a principal goal of the present studies was realized, namely, the isolation of an enriched subpopulation of OA-sensitized T lymphocytes. The day 9 B-band cells capable of giving a proliferative response upon challenge with OA in culture must be regarded as OA-specific T memory cells because a) the proliferative response to OA is obliterated by treatment with T-specific antiserum plus complement, along with responses to PHA and ConA; b) these cells exhibit no surface Ig by the immunofluorescence technique; c) histologic studies demonstrate massive hypertrophy of the thymus-dependent paracortical areas and the absence of germinal centers in the inguinal lymph nodes at this time, precluding the possibility that germinal center B-lymphocytes, also of light to moderate density, might be present in the B-band; and d) these cells produced high concentrations of lymphotoxin on challenge with specific antigen. Careful counts of blasts developing within the first 24 hr established that 20 to 30% of these cells must be regarded as specifically responsive to OA.

In contrast to the above, when cells of band C at day 17, principally responsible for the second rise in OA responsiveness at this time, are treated with T-specific antiserum plus complement, the response to OA is enhanced. Many germinal centers now are present in the inguinal nodes. Further, a significant proportion of the cells in bands B and C, which were entirely fluorescence-negative for membrane immunoglobulin on days 6 and 9 after sensitization, now show positive staining. It is probable, therefore, that many or perhaps all of the responsive cells at this time are B lymphocytes, either in or migrating from the germinal centers.

Although the T cell-specific antiserum lysed only 56% of the B-band cells at day 9, it abolished the responses to OA, PHA, and Con A. The remaining cells were immunoglobulin-negative

by the fluorescence technique. These could be either T or B cells in the course of differentiation and proliferation, altered in some way which diminished their reactivity with the T cell-specific and Ig-specific antisera, or, alternatively, some type of "null" cell (27). It is doubtful that their negative immunofluorescence results from internalization of label, since assays on several subpopulations were run simultaneously and there were, therefore, always adequate positive controls.

Since macrophage participation is obligatory for the triggering of T cell DNA synthesis by soluble antigens (23, 28, 29) and for production of lymphotoxin (29) and enhances the response to mitogens such as PHA and Con A (30-32), the role of the macrophage in the presentation of antigen to the T cell in these studies must be considered. Addition of purified macrophages to the various cultures throughout these studies failed to affect the levels of response observed. It would seem then that the differences in reactivity of different bands observed is real and not simply due to relative changes in macrophage concentration.

Our result agrees with the description of lymphocytes recently immunized to allogeneic erythrocytes, histocompatibility, tumor, or microbial antigens as being of low to moderate density. However, in our experiment, the large cells of low density in the B-band at 9 to 12 days, which have recently responded to an antigenic stimulus, are capable of reinitiating DNA synthesis in response to the same antigen, although lymphocytes sensitized to histocompatibility antigen (T. TDL) studied by Sprent and Miller cannot (2, 10, 11). Of remarkable significance is the fact that B-band cells apparently are unable to respond to either PHA or Con A. Lymphotoxin production with OA also was confined to cells of light and intermediate density on day 9, the same fraction responding to OA with DNA synthesis; these cells did not produce lymphotoxin when PHA was the stimulant. Of further interest is the fact that in our system, the sensitized T cells of band B capable of giving a proliferative response to OA at day 9 proved to be adherent to glass wool, whereas the subpopulation responsive to PHA and Con A remained nonadherent or slightly adherent throughout (manuscript in preparation).

Heterogeneity among T lymphocytes is well recognized and has been described in terms of surface antigens, functional studies, physiologic attributes, and recirculation patterns (reviewed in 33). Our data are in agreement with those of Stobo and Paul (34) who report that cells responsive to Con A are mainly located in fractions of intermediate density when separated on BSA gradients, whereas PHA-responsive cells are concentrated in denser lymphocyte fractions. The OA-sensitized T cells found in enriched numbers in band B at day 9 proved to be lighter than either of these, although OA-sensitized T cells were present across the range of densities examined. The question of T cell heterogeneity may be extended to possible subsets within the latter subpopulation. B-band cells on day 9 which proliferate in response to OA are adherent cells whereas those which produce lymphotoxin may be nonadherent to glass wool (29). There is no evidence in the present experiments to indicate whether these are in fact the same cells or separable subpopulations. Both may represent differentiation states of cells responsive to OA which initially had the additional capacity to respond to the standard T mitogens. Their new surface properties would result from a loss of PHA or Con A-binding sites or possibly a change in the excitation threshold of these sites. Cytochalasin B in low concentrations strikingly potentiates the responses of T cells to PHA or to

suboptimal concentrations of Con A. In contrast, the response of sensitized cells to antigen is not potentiated, an observation favoring the second suggestion (35, 36). It is also possible that cells responding to stimulation with specific antigen have a different lineage, i.e., precursors that did not react to mitogens at any time during their life history, since the functional tests applied herein suggest that the OA-sensitized lymphocyte does not respond to PHA, either by blastogenesis or by lymphotoxin production. The low mitogen responses elicited from cells of the B-band would then represent responses by contaminant cells from other populations.

The fact that increased cell yields were noted first in band B on day 5, followed by band C on day 7, and lastly by D and the pellet, agrees with published descriptions that, after sensitizing exposure to antigen, the immunocompetent cell undergoes blast transformation and repeated cell division, then diminishes in size (1, 13, 17). It is of interest, however, that, although cell yields for the various bands increased in the anticipated order, proliferative responses to OA in culture did not occur first in band B on day 5 but, rather, in band D. Since it is possible to elicit a proliferative response from cells of band B on day 9, it is apparent that larger, less dense cells, having passed the peak of their differentiation, are capable of being restimulated. Therefore, on days 5 and 7, the responses to OA by cells of D, and C and D, respectively, appear to be out of synchrony. Perhaps, cells of B and C at days 5 and 7 are committed to other functions (e.g., lymphokine production). There is a wave of early suppressor cell activity in animals given antigen (37) and there may be preferential suppression of the responses of larger cells exposed to antigen. It is also possible that too few sensitized cells are present in bands B and C at this time to permit detection via the proliferative response assay.

The total PHA response increased dramatically by day 9. This does not represent an increase in the responsiveness of individual cells but, rather, the increased numbers of T cells in the inguinal nodes at this time. The large numbers of PHA-reactive cells appearing at the peak of the lymph node response to antigen must depend largely on recruitment from the recirculating pool of T cells by some trapping mechanisms (38, 39), possibly mediated by factors released from the specifically reacting cells (40). It may also involve local proliferation of such cells in response to the same factors or to lymphocyte-activating factor formed by macrophages locally in response to the injected adjuvant (31).

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